HHS Public Access

Author manuscript

Leukemia. Author manuscript; available in PMC 2020 February 28.

Published in final edited form as:

Leukemia. 2019 December ; 33(12): 2962–2966. doi:10.1038/s41375-019-0528-3.

Phorbol ester induced *ex vivo* expansion of rigorously-defined phenotypic but not functional human cord blood hematopoietic stem cells: a cautionary tale demonstrating that phenotype does not always recapitulate stem cell function

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Hematopoietic stem cells (HSCs) are a rare population residing at the apex of the hematopoietic hierarchy [1]. HSCs have the capacity to self-renew and differentiate into all blood cell types, thus they play a key role in hematopoietic cell transplantation (HCT) [2]. HCT is widely used as a curative therapy for numerous malignant and non-malignant hematological and even non-hematological diseases [3]. The fast developing field of gene editing techniques, including ZFNs, TALENs and CRISPR-Cas9, broaden usage of HCT in clinical therapy of diseases caused by genetic mutations [4]. i.e. β-thalassaemia or Sickle Cell Disease (SCD) may possibly be interrogated by CRISPR based gene editing of β-globin in HSCs from patients [5]. However, efficient gene editing and infusion of the gene edited

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Author contributions

YC, CY, BG and XH performed the experiments and data analysis. YT and RJ collected blood samples and performed data analysis. SL and JW performed RNA-seq data analysis and functional enrichment analysis. BG and HEB designed and supervised this study. All authors had access to the final draft manuscript and approved the submission of the article.

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Conflict of interest

The authors declare that they have no conflict of interest.

HSC into patients requires sufficient numbers of donor HSC. In addition, when cord blood (CB) is used as the source of HSC for HCT, rare HSC numbers in single CB units may lead to delayed hematopoietic recovery in recipients [6]. It is thus important in some cases to develop efficient means that can overcome limited HSC numbers to enhance the efficacy of HCT.

Efforts are underway by many scientific groups to develop potential ways to improve engraftment of HSC. This includes mitigating EPHOSS [7], enhancing HSC homing efficiency [8–9], promoting ex vivo expansion [10–12], and by reprogramming iPS or endothelial cells to human HSC [13–14]. However, most of these methods have not yet been adapted for clinical use, and there is still an urgent need to dissect the mechanisms behind these encouraging processes. Rigorous phenotypic analysis of HSC is an easily accessible and important method to evaluate the function of a heterogeneous cell population from bone marrow or blood samples. The rigorous phenotyping identity of human HSC identity was confirmed at a single cell level [15]. In general, freshly isolated human HSC are defined as $CD34^+CD38^-CD45RA^-CD90^+CD49f^+$. However, we still don't know if all cells in different circumstances, especially under stress conditions such as ex-vivo expansion, that are identified by $CD34^+CD38^-CD45RA^-CD90^+CD49f^+$ are functional HSC with long-term repopulating capacity.

In a compound screen for agonists of human CB HSC ex vivo expansion, we found that 100 nM 12-deoxyphorbol-13-O-phenylacetate-20-acetate (DOPPA), a weak phorbol ester, significantly and greatly expands cytokine (stem cell factor (SCF), thrombopoietin (TPO), Flt-3 ligand (FL)) -stimulated phenotypic CB HSC population ($CD34^+CD38^-CD45RA^-CD90^+CD49f^+$) (Figure 1A). Compared with the vehicle control cultured group, the DOPPA group contains 12.4-fold more phenotypic-defined HSCs after 4-days ex vivo culture (Figure 1B). The phenotypic HSC number in DOPPA-treated group was ~2.5 fold greater than that seen the SR1 and UM171-treated groups at this 4-day culture time (Figure 1B).

We then examined the effect of DOPPA treatment on hematopoietic progenitor cells (HPCs). DOPPA significantly increased numbers of CB colony-forming unit (CFU) granulocyte/macrophage (GM), but not granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) progenitors after 4 days *ex vivo* culture (Figure 1C), suggesting that DOPPA treatment selectively promotes *ex vivo* expansion of functionally recognizable granulocyte-macrophage progenitors.

In order to determine if the DOPPA expanded phenotypic HSC population had repopulating or increased repopulating capacity in vivo, we performed limiting dilution analysis to quantify SCID-repopulating cells (SRCs) by intravenous (i.v.) injection in sublethally-irradiated NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}*/SzJ* (NSG) mice. The SRC frequency of DOPPA -cultured CB $CD34^+$ cells and human $CD45^+$ cell chimerism did not significantly increase compared with that of uncultured $CD34^+$ cells and vehicle control-treated $CD34^+$ cells (Figure 1D–F; Supplementary Table 1), whereas numerous studies by others,[10,11] and ourselves (unpublished data) have shown significantly enhanced SRCs after ex-vivo

expansion with SR1 and UM171. These data strongly suggest that the DOPPA-expanded phenotypically-defined HSC were not active as engrafting HSC.

Interestingly, we noticed that phenotypic HSC were significantly increased if we cultured the $CD34^+$ cells just for 24 hours with a higher concentration of DOPPA (1μM) or other phorbol esters, including phorbol-12-myristate-13-acetate (PMA) (Supplementary Figure 1A-B). We performed *in vivo* transplantation experiments to examine if PMA-induced phenotypic HSC have long-term engrafting capacity. 10,000 vehicle or PMA-cultured $CD34^+$ cells were transplanted into sublethally-irradiated NSG mice. The human cell engraftment in the BM of the NSG recipients was determined 16 weeks after transplantation. Our data showed that $CD34^+$ cells cultured with DOPPA or PMA did not engraft better than the cells cultured with vehicle control (Supplementary Figure 1C-D). To exclude the possibility that PMA might affect the homing efficiency of $CD34^+$ cells, we did homing experiments to examine the human cell percentage engrafted into the bone marrow of the recipient NSG mice 24 hours after transplantation. We found that PMA treatment did not significantly change the homing efficiency of CB HSC and HPC (Supplementary Figure 2A-B). Together, these data demonstrate that the PMA-induced phenotypic HSC after *ex vivo* culture with cytokines were not capable of functional engraftment with long-term repopulating activity.

To understand mechanisms behind the unusual inconsistency between *ex vivo* HSC phenotyping analysis and *in vivo* functional evaluation, we did RNA-sequencing analysis to check the transcriptome of vehicle and DOPPA-treated $CD34^+$ cells after 4 days ex-vivo culture. We noticed that expression of many genes which encode cell surface proteins including *ITGA6* (*CD49f*) were significantly up-regulated, FDR-adjusted p-value < 0.01 and the fold change (FC) > 2, by DOPPA treatment (Figure 2A). Gene ontology (GO) analysis revealed that many GO functions related to cell surface or plasma membrane related GOs which all include ITGA6 were significantly enriched in up-regulated differentially expressed genes (DEGs) including ITGA6 after DOPPA treatment (Figure 2B). Quantitative PCR analysis further demonstrated that *ITGA6* mRNA levels apparently increased in DOPPA-treated $CD34^+$ HSCs and HPCs (Figure 2C). We also checked mRNA expression of *THY1*(*CD90*), another marker of human HSC, the signal of which was not detected by RNA-seq analysis due to its low abundance. The *THY1* mRNA level was largely increased in DOPPA treated $CD34^+$ HSCs and HPCs (Figure 2C). Flow cytometry analysis showed that *ITGA6* and *THY1* surface expression was largely promoted by DOPPA (Supplementary Figure 2C-D). Increased *ITGA6* expression in DOPPA treated $CD34^+$ cells was further confirmed by T-distributed Stochastic Neighbor Embedding (tSNE) analysis (Figure 2D). These data indicated that DOPPA treatment promoted transcription and cell surface expression of *ITGA6* and *THY1*, and thus had changed the percentage of the phenotypically-defined cells during *ex vivo* culture. We also found many genes involved in myeloid differentiation that were significantly enriched by DOPPA (Supplementary Figure 2E-F), consistent with the increased numbers of CFU-GM we had detected (Figure 1C). These data may partly explain the DOPPA treatment-induced expansion of CFU-GM.

Phenotypic analysis of HSCs is a widely used approach to relatively quickly sort or quantify numbers of HSC, allowing for a rapid read-out of these cells. However, not all published

studies have focused on the correlation between the phenotyping and reconstituting capacity of the cells. Phorbol ester short treatment significantly changed the transcription and expression of cell surface markers used to define HSC, including *ITGA6* and *THY1*. Our findings provide an excellent example for investigators in this field that it is crucial for full analysis of effects on HSC function that engrafting studies always be performed to confirm functional activities, as phenotype may not always recapitulate function. The quantification of HSC is based on expression of several cell surface markers. In some circumstances, mRNA expression needs to be considered to exclude the possibility that the transcription of those genes encoding HSC markers were changed by the treatment. Future study will likely need to be performed to identify candidate HSC markers that can reflect the reconstituting capacity of the cells in different circumstances, especially under stress conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

These studies were supported by the following Public Health Service grants from the US National Institute of Health to HEB: R35 HL139599, R01 DK109188, R01 HL056416, and U54 DK106846, and by start-up grants to BG from Shanghai Jiao Tong University School of Medicine. The bioinformatics analysis was performed by the Collaborative Core for Cancer Bioinformatics (C3B) shared by Indiana University Simon Cancer Center (P30CA082709) and Purdue University Center for Cancer Research (P30CA023168) with support from the Walther Cancer Foundation.

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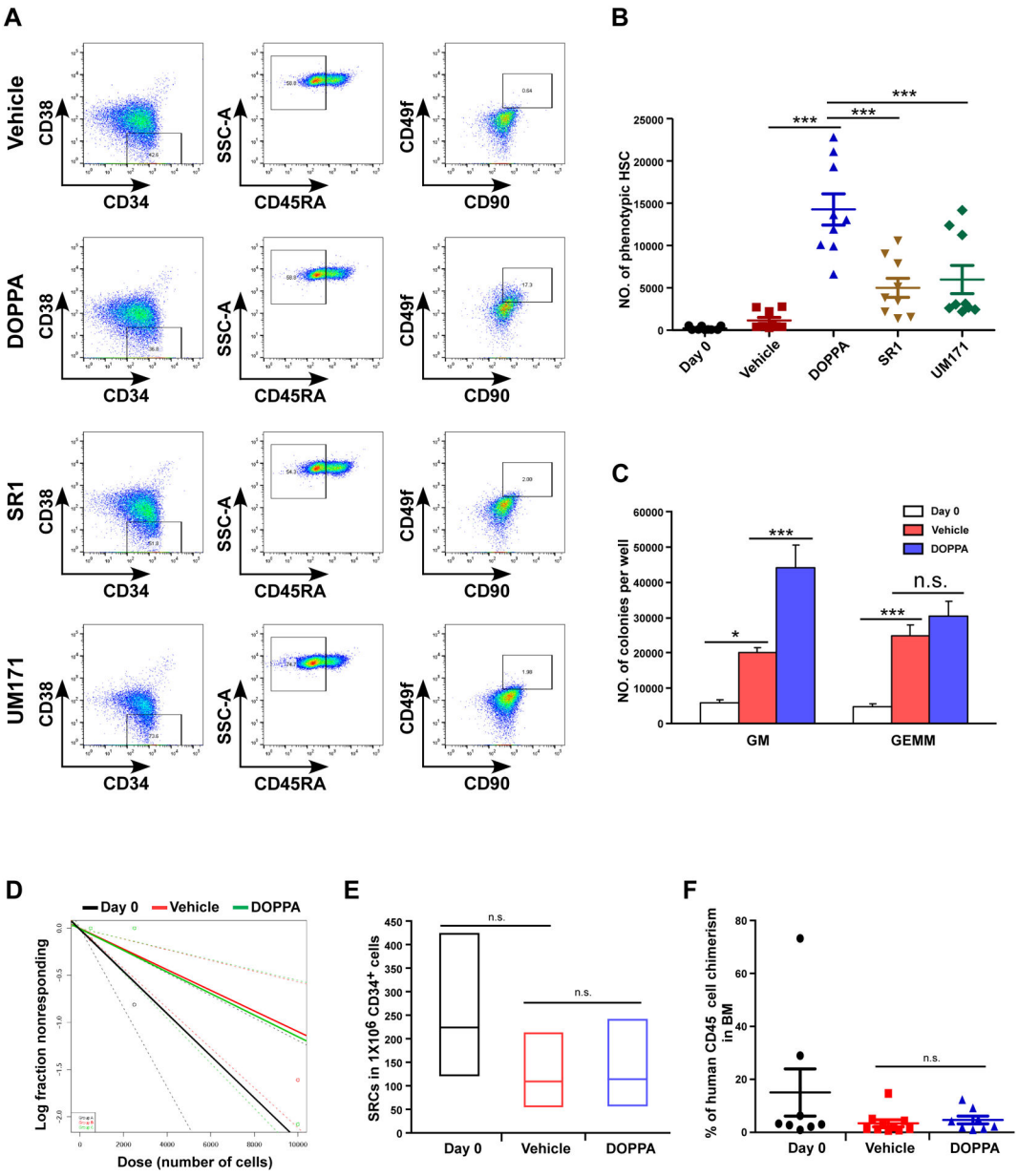

Figure 1. DOPPA expands human cord blood phenotypic hematopoietic stem cells without repopulating capacity.

(**A**) Representative FACS plots (from n = 3 independent experiments) showing *ex vivo* expansion of cord blood (CB) phenotypic hematopoietic stem cells (pHSCs) after treatment with vehicle (DMSO), DOPPA (100 nM), SR1 (1μM), UM171 (100 nM) for 4 days. The pHSC population was defined as $CD34^{+}CD38^{-}CD45RA^{-}CD49f^{+}CD90^{+}$ cells.

(**B**) Quantification of pHSCs expanded by vehicle (DMSO), DOPPA (100 nM), SR1 (1 μM), or UM171 (100 nM) at day 4. Data are shown as mean±s.e.m.

(**C**) Quantification of CFU numbers in 50,000 $CD34^{+}$ cells (for each well of the culture plate) of day 0 uncultured cells and the progeny of an equivalent number of $CD34^{+}$ cells expanded by vehicle or DOPPA for 4 day (n = 9 cultures from three independent experiments per group). Data are shown as mean±s.e.m.

(**D**) Poisson distribution analysis plot of the frequency of human SRCs in uncultured CB $CD34^+$ cells (black line; group A) or in the progeny of an equivalent number of $CD34^+$ cells that were cultured with vehicle (red line; group B) or DOPPA (green line; group C) for 4 d. (n = 7 to 10 mice per group). Circles represent the percentage of negative mice for each dose. Solid lines indicate the best-fit linear model for each data set. Dashed lines represent 95% confidence intervals.

(**E**) SRCs (line in the box) in 1×10^6 $CD34^+$ cells for each group and the relative 95% confidence intervals (box) are shown. L-Calc™ Software was used for Poisson statistical analysis. n.s. marks non-significant difference.

(**F**) Human $CD45^+$ cell chimerism in the bone marrow of NSG recipient mice 4 months after transplantation. Data are shown as mean±s.e.m. One-way ANOVA followed by Tukey test was performed in figures (B), (C) and (F). *$p<0.05$; ***$p<0.001$.

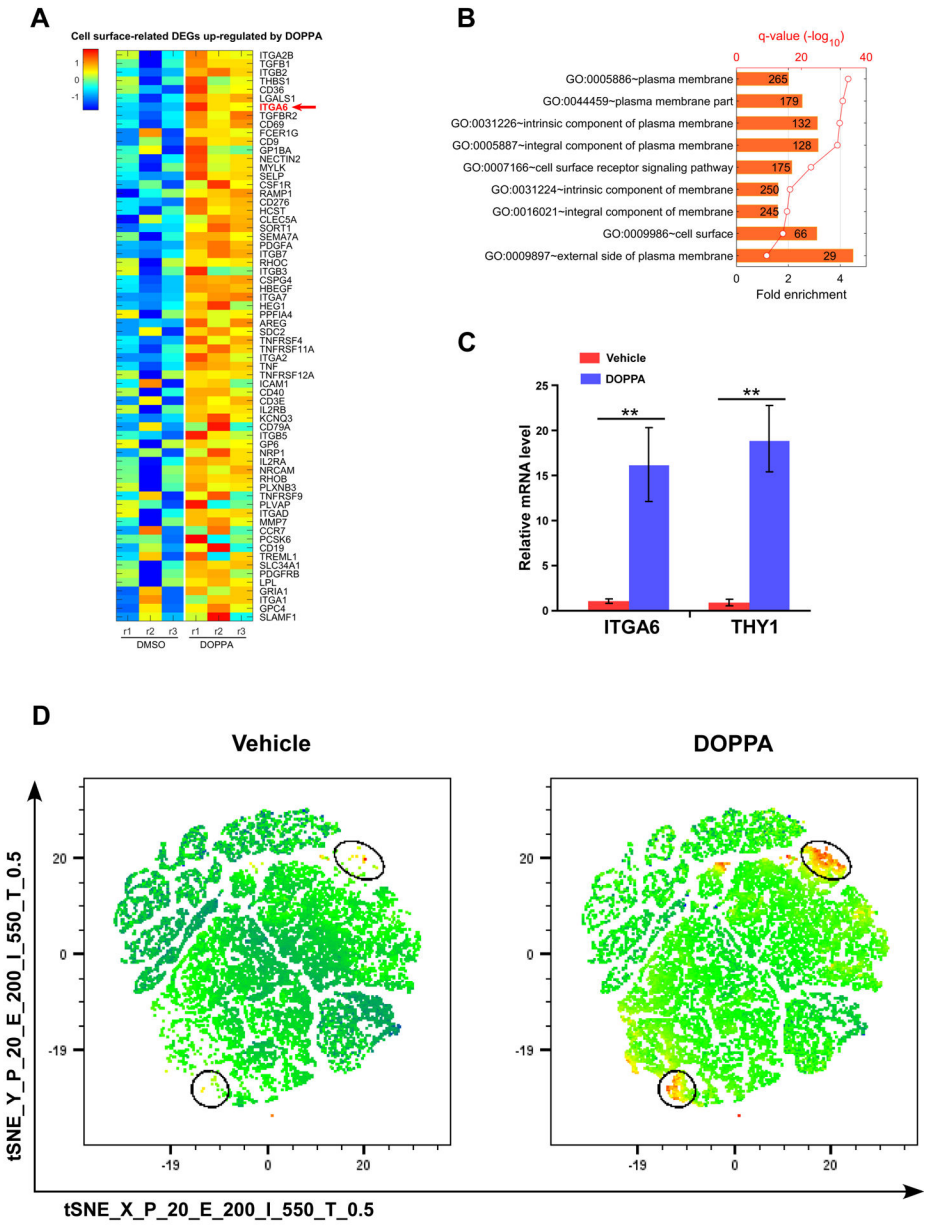

Figure 2. DOPPA treatment promotes transcription of genes encoding cell surface proteins including *ITGA6* and *THY1*.

(**A**) Heat map showing differentially expressed genes (DEGs) associated with “cell surface” up-regulated by DOPPA treatment. Each group has three replicates (r1, r2, r3).

(**B**) Selected GOs that are significantly enriched in DOPPA-treated group compared with vehicle control group.

(**C**) Quantitative real-time PCR analysis of *ITGA6* and *THY1* expression in vehicle or DOPPA treated $CD34^+$ cell. Data are shown as mean±s.d. Student’s t-test was performed. **p<0.01.

(**D**) tSNE analysis showing *ITGA6* expression in vehicle or DOPPA treated $CD34^+$ cells. CD34-APC gate was selected as the input. tSNE analysis was performed using FlowJo_V10

software to compare *ITGA6* expression in vehicle or DOPPA treated groups. The circles indicate $CD34^+$ cells with high levels of *ITGA6*.